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13. ABSTRACT (Maximum 200 Words)

Study Design: The proposed study will first evaluate the role of Akt, in protection against doxorubicin and anti-erbB2- cardiomyocyte toxicity, using adenoviral expression of active Akt, pharmacological inhibitors of this pathway, and two peptides that activate Akt, cardiotrophin-1 and urocortin. Since these peptides have not been reported expressed in breast tissues or cancer, to confirm this, we will evaluate the expression of both peptides and their receptors in six commonly studied breast cancer cell lines and 160 breast cancer tissue arrays by immunohistochemistry and western blotting methods. Even if expression is observed in breast tissue, peptide treatment may improve cancer therapy as seen in other models. In aim 3, the cardiotrophin-1 and urocortin cardiac protection strategy, will be tested against cardiac toxicity induced by doxorubicin, anti-erbB2, chemical inhibitors of erbB1 or erbB2, or combination treatments. This will be a direct comparison of rat and human cardiomyocytes with 6 breast cancer cell lines using MTT assay. Next both peptides, will be administered in pilot studies to Sprague Dawley rats to establish a dose that protects against doxorubicin induced cardiac toxicity. Finally, using a female nude rat breast cancer xenograph model, these peptides will be evaluated for specific cardiac protection, during treatment with doxorubicin, anti-erbB2, combination of doxorubicin and anti-erbB2 and controls. Echocardiography, to evaluate ejection fraction, white blood cell counts, to evaluate bone marrow toxicity, histopathology, xenograph tumor size and weights will be used to assess peptide cardiac specific protection and anti-neoplastic therapy.

Relevance: Doxorubicin is currently a first choice drug for breast cancer treatment, limited in use by its cardiac toxicity. Combination drug treatment is the standard of care. This proposal addresses a timely clinical problem observed with doxorubicin and anti-erbB2 combined therapy, as well as the potential problem of combined treatment with doxorubicin with other erbB inhibitors. This strength of this proposal is the direct comparison of relevant human cardiomyocytes and human breast cancer cells to better understand the mechanism of toxicity and to evaluate in human cells, a novel peptide protection strategy aimed to reduce cardiac toxicity without affecting breast cancer anti-neoplastic therapy.

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Table of Contents

Progress report

Kathleen Gabrielson

AKT RESCUE IN CARDIOMYOCYTES BUT NOT BREAST CANCER CELLS AFTER DOXORUBICIN AND ANTI-ERBB2 TREATMENT

Introduction

Significance of the project:

Anti-erbB2 (Herceptin) and doxorubicin are effective treatments for breast cancers that over-express the Her2/neu oncogene. In pivotal trials that lead to its approval, anti-erbB2 administered in combination with other agents, particularly doxorubicin, 29% of patients developed cardiac dysfunction, and in some cases fatal, cardiomyopathy [1-3, 10]. The mechanism of this synergistic toxicity induced by anti-erb2 is not understood. To address this problem, we have developed novel *in vitro* and *in vivo* rat animal models that exhibit cardiac toxicity synergism with doxorubicin and anti-rat erbB2 compared to either treatment alone. We utilize an anti-rat-erbB2 monoclonal antibody (clone 7.16.4) that shows the same biological effects in rat cells expressing erbB2, as Herceptin does in human breast cancer cells, including similar epitope recognition, inhibition of cell growth, reversion of phenotype and reduction in cancer cell growth *in vivo* [4]. In this model, Akt, a well-known anti-apoptotic pathway protein in the heart, linked to the erbB2, is inactivated. This finding of Akt inactivation is consistent with Herceptin's mechanism of action in breast cancer cells [5, 6]. Cardiotrophin-1 and urocortin both stimulate the Akt pathway through different receptors expressed in the heart [7-9], and not breast cells; thus, treatment with these peptides, may circumvent the erbB-linked Akt pathway and provide protection during doxorubicin or doxorubicin/anti-erbB2 treatment.

The hypothesis of this proposal is that activation of Akt in the heart through heart specific receptors, during doxorubicin and anti-erbB2 therapy, will protect from cardiac toxicity and not diminish doxorubicin and anti-erbB2 tumor cell killing in breast cancer cells.

Specific Aim 1 Determine the role of Akt activation, by non-erbB2 pathways, in protection of cardiomyocytes against toxicity induced by anti-erbB2, doxorubicin, chemical inhibitors of erbB1 or erbB2 or combination treatments.

Specific Aim 2 Screen a panel of commonly used human breast cancer cell lines, breast cancer tissue arrays and normal epithelium arrays for expression to urocortin or cardiotrophin-1 peptides or their receptors.

Specific Aim 3 Determine whether activation of Akt, by the non-erbB2 pathways, (urocortin or cardiotropin-1), will preferentially protect cardiomyocytes and not breast cancer cells, against toxicity induced by anti-erbB2, doxorubicin, inhibitors of erbB1 or erbB2 or combined treatments.

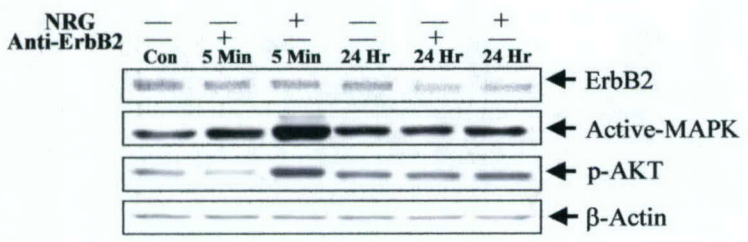
Specific Aim 4 Determine whether in vivo pretreatment with Akt-inducing urocortin or cardiotropin-1 provides protection for doxorubicin and /or anti-erbB2 treatment induced cardiac toxicity in rats without affecting anti-neoplastic effects of therapy.

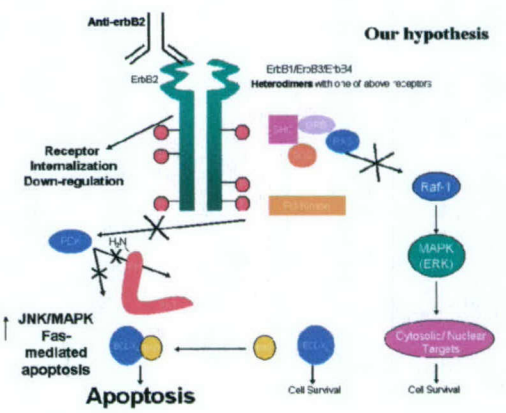
Body: Research Accomplishments

To test our hypothesis, our specific aims are:

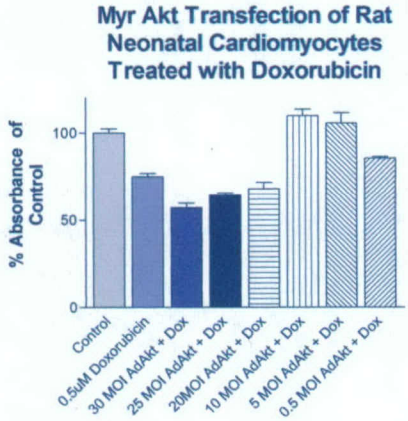
Specific Aim 1 Determine the role of Akt activation, by non-erbB2 pathways, in protection of cardiomyocytes against toxicity induced by anti-erbB2, doxorubicin, chemical inhibitors of erbB1 or erbB2 or combination treatments.

Anti-erbB2 reduces activation of Akt and ERK1/2MAPK compared to NRG

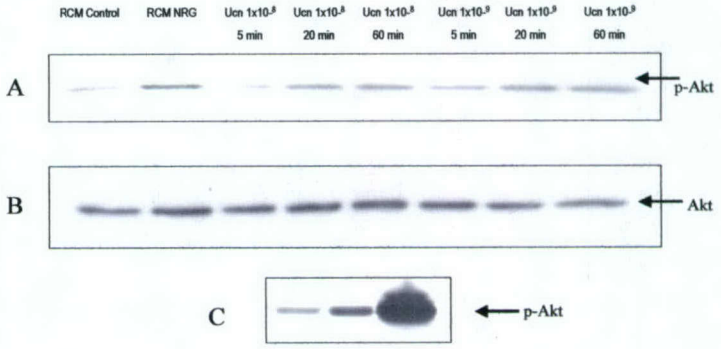




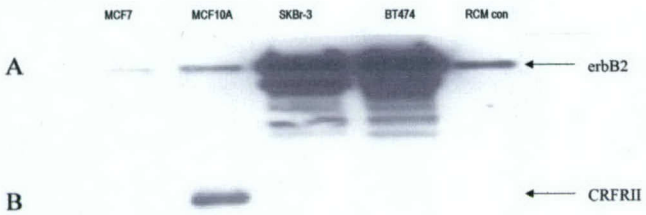
An Akt expressing adenovirus induced protection against doxorubicin. MOI of 5-10 showed the best protection (MTT assay below left). Below right (A) Western blot from urocortin-treated cardiomyocytes lysates. The amount of phosphorylated Akt increases as urocortin concentration and exposure time increase. (B) The amount of Akt stays the same as urocortin concentration and exposure time increase. (C) When cardiomyocyte lysates were treated with an adenovirus that expresses active Akt, the amount of Akt in the cells increased lane 3, lane 2 NRG treatment, lane 1 control.



Cardiomyocytes treated with an adenovirus that expresses active Akt are protected from cardiac toxicity. (n=12)



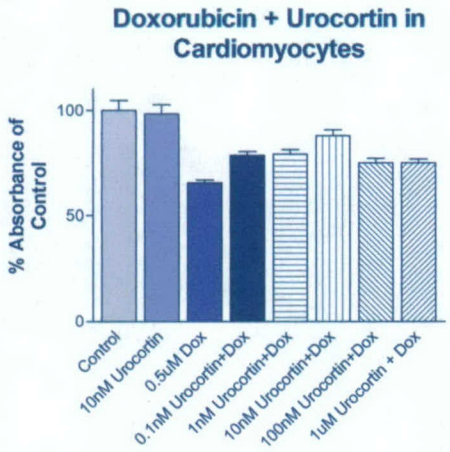
Specific Aim 2 Screen a panel of commonly used human breast cancer cell lines, breast cancer tissue arrays and normal epithelium arrays for expression to urocortin or cardiotrophin-1 peptides or their receptors.



Cell Lines	Urocortin	CRFRII	ErbB2
BT474	No expression	No expression	High expression
MCF7	No expression	No expression	Low expression
MCF10A	No expression	Moderate expression	Low expression
SKBr-3	No expression	No expression	High expression

The previous blot compares protein expression between RCM (rat neonatal cardiomyocytes, control) and various breast cancer cell lines. The antibody to CRFRII only cross reacts with the human protein and not the rat protein. At present, there is not a suitable antibody for the rat. The expression of urocortin and its receptor, CRF2 are summarized in the above table. It is known that urocortin does protect cardiomyocytes from ischemia/reperfusion conditions. Since urocortin treatment offered protection to cardiomyocytes, we screened a panel of commonly used human breast cancer cell lines for the expression of urocortin and its receptor, CRFRII. We next determined whether activation of Akt by the non-erbB2 pathway, urocortin, would preferentially protect cardiomyocytes and not breast cancer cells against toxicity induced by doxorubicin. Results are presented below.

Specific Aim 3 Determine whether activation of Akt, by the non-erbB2 pathways, (urocortin or cardiotropin-1), will preferentially protect cardiomyocytes and not breast cancer cells, against toxicity induced by anti-erbB2, doxorubicin, inhibitors of erbB1 or erbB2 or combined treatments.



Above Left figure: The number of metabolically active neonatal rat cardiomyocytes was determined by the MTT assay. Neonatal rat cardiomyocytes were treated doxorubicin and urocortin. Urocortin protects neonatal rat cardiomyocytes from cardiac toxicity. (n=36)

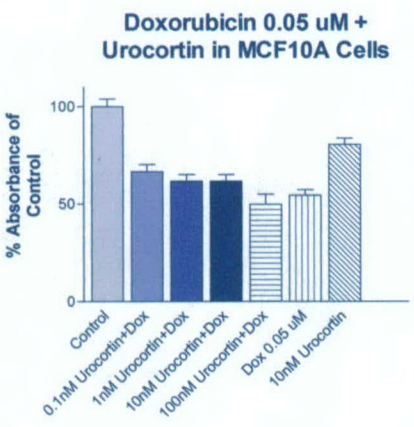


Figure (left) The number of metabolically active MCF10A cells was determined by the MTT assay. MCF10A cells were treated Doxorubicin (0.05uM) and Urocortin (variable concentrations). Urocortin does not protect MCF10A cells from doxorubicin toxicity. (n=12)
This suggests that stimulation of other Akt pathways, may be protective with dual doxorubicin and anti-erbB2 treatment in the heart but not in breast cells that have a receptor. Future studies will test if urocortin can offer protection in this combined treatment in cardiomyocytes.

Figure 10: Western blots from urocortin treated MCF10A lysates (10nM for 20 minutes). The amount of phosphorylated Akt in the cells does not change as urocortin concentration increases. RCM=rat neonatal cardiomyocytes, NRG= neuregulin 1β, positive control for Akt activation

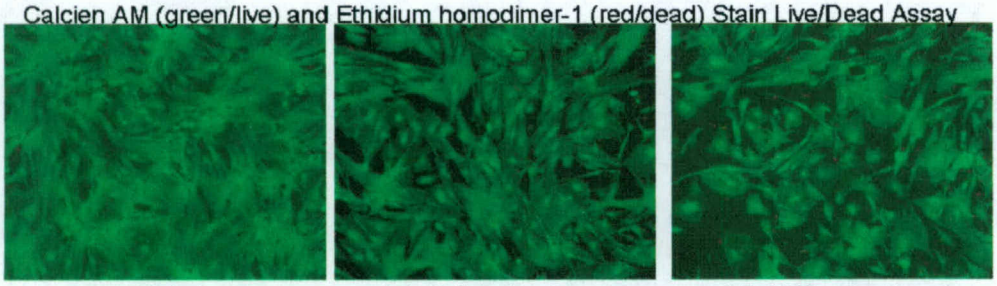
Figure 11: Western blots from MCF10A lysates treated with urocortin (10 nM) for varying times. The amount of phosphorylated Akt in the cells does not change as exposure time increases. RCM=rat neonatal cardiomyocytes, NRG= neuregulin 1β, positive control for Akt activation

The following is validation of two cytotoxicity assays with doxorubicin which will apply to Aim 1 and 3: These two assays will be used in doxorubicin and erbB2 inhibition, as well as PI3K inhibition experiments. These assays will also be necessary to use in doxorubicin toxicity prevention experiments. Our prior experiments used the MTT assay which measures mitochondrial function and not always correlated with cell death (multiple reviewer's comments). We will next analyze how MTT, LDH and the Live/Dead cell assays

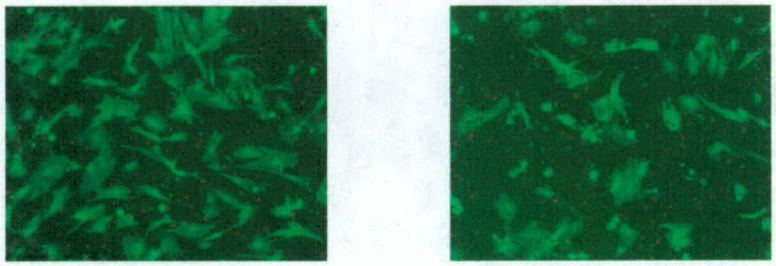
correlate by doing linear regression statistics, as was done with the validation of LDH and the Live/Dead cell assay compared in the below figure.

Dose/Response

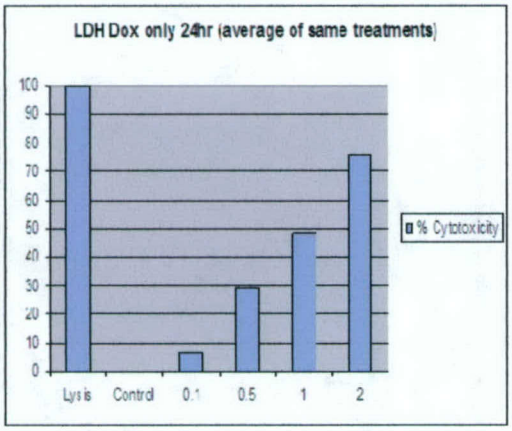
Calcien AM (green/live) and Ethidium homodimer-1 (red/dead) Stain Live/Dead Assay

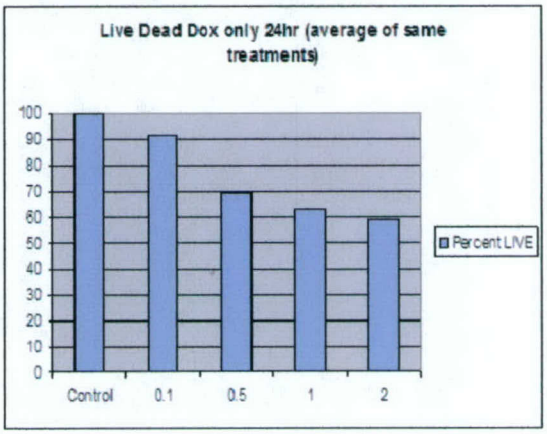


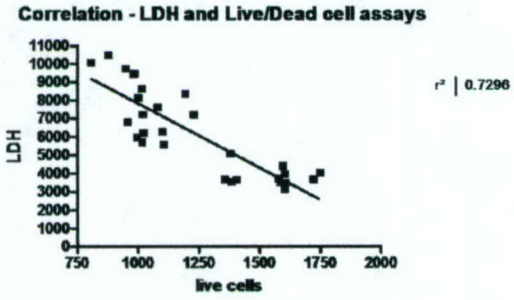
Control | 0.1uM Dox treatment | 0.5uM Dox treatment



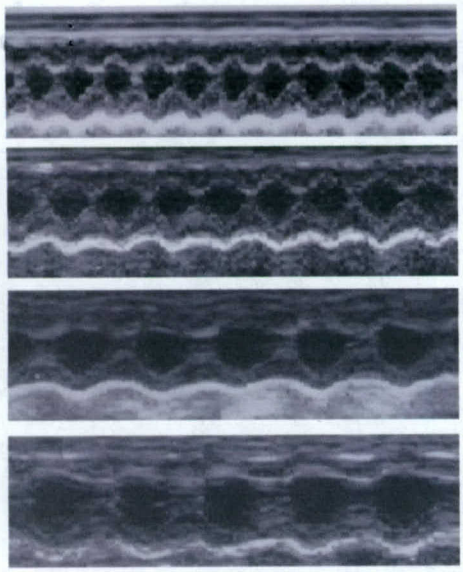
1.0uM Dox treatment | 2.0uM Dox treatment

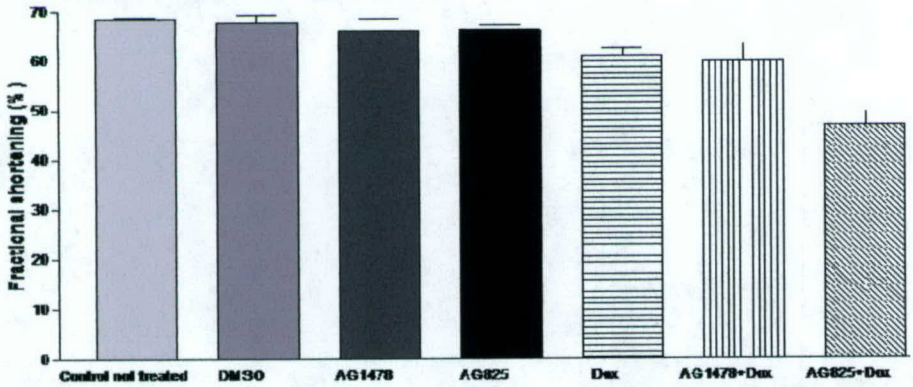






This year we also developed a mouse model of chronic doxorubicin toxicity. Below is the M-mode of the left ventricle over time. This figure shows the progressive dilation and loss of cardiac contractility with administration of (4) tail vein intravenous injections of 9 mg/kg doxorubicin given every 2 weeks.





Using this same model, but using only 3 tail vein intravenous injections of 9 mg/kg doxorubicin given every 2 weeks, we applied the concept in Specific Aim 4 by inhibiting erbB1 and erbB2. Giving a combined treatment of Doxorubcin and AG825, an erbB2 inhibitor, synergistic toxicity was observed resulting in a marked reduction in the cardiac fractional shortening evaluated by in vivo transthoracic echocardiography. This model will be evaluated for protection with urocortin, an pAKT inducer along side the rat model.

Monoclonal antibody production: We require a large amount of antibody to perform the anti-erbB2 in vitro and in vivo experiments. We had a three month period when we could not make antibody because of a technical problem that was finally solved. This had an impact on rat studies, although the mouse model listed above may be added if we can not make enough antibody for these complete studies. I will see what can be accomplished this year before changing the protocol. The hybridoma we use to produce 7.16.4 has always been grown in an Integra flask (Integra Biosciences). We have two flasks going at a time to make about 5mg/week. In order to meet the large quantity of antibody we need for these animal studies, we also set up a Hollow fiber system HFS (Bellco) to increase antibody production. Our antibody is routinely checked for activity by flow cytometry and western blotting (erbB2 phosphoryation). Each rat in a study requires 4mg/study. We were unsuccessful in adapting our hybridoma to the HFS, even with close consultation with the company's president. Some hybridomas are not adaptable to the HFS.

Key Research Accomplishments

- **Akt expressing Adenovirus protects against doxorubicin toxicity in cardiomyocytes**
- **Urocortin inducers AKT activation (pAKT) in cardiomyocytes**
- **Comparison of erbB2 and CRF2 expression in breast cancer cell lines and cardiomyocytes**
- **Only MCF10A cells have CRF2 receptor for urocortin**
- **Urocortin provides protection against doxorubicin toxicity in cardiomyocytes**
- **Urocortin does not provides protection against doxorubicin toxicity in MCF10A cells**
- **Validation of two cytotoxicity assays for this project**
- **Inhibition of erbB2 in a mouse model of doxorubicin toxicity induces synergistic cardiac toxicity**

Reportable Outcomes

Manuscripts

Gabrielson KL, Becker R, Shi W, Servinsky M, Bedja D, Barber S, Akao M, Peterson N. Synergistic cardiac toxicity with doxorubicin and anti-erbB2 treatment in rats: Model for Herceptin-induced cardiac toxicity. In revision

Presentations

JHU- Department of Oncology Breast cancer research SPORE program July 2004, "Breast cancer therapies that induce cardiac toxicity- Can cardiac Akt activation prevent?"

JHU-ICMIC P50 program Department of Radiology September 2004, "Role of Akt pathway in immuno and chemotherapy for breast cancer"

University of Colorado, Denver, School of Medicine, Department of Oncology "Synergistic toxicity induced by doxorubicin and anti-erbB2 in rat model", January 2005

Conclusions

In the first year of this project, we have accomplished several of the proposed experiments in the Statement of Work in Aims 1-3. Activated AKT is protective during doxorubicin toxicity in cardiomyocytes. We identified a peptide (urocortin) that also provides protection during doxorubicin toxicity in cardiomyocytes. Most importantly, we screened several breast cancer cell lines and did not find expression of the receptor for this peptide. Thus, this peptide is a candidate to test in vivo for protection against doxorubicin and the combined anti-erbB2/doxorubicin treatment.

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Appendices

None to submit